FDSS7000EX FDSS/µCELL

Lonza

New Tools for Drug Discovery: Multiplexing the monitoring of Ca^{2+} and cAMP signaling in primary cells using the FDSS®/µCELL.

Introduction

In drug discovery research cell-based assays are a widely accepted tool for high throughput screening of potential drug candidates. So far primary cells are not being used very frequently although they originate from the actual human tissue of interest, genuinely express the relevant drug targets, e.g. cell surface receptors at physiological levels, and carry the endogenous cofactors necessary for efficient and specific signal transduction. Thus, primary cells resemble the in-vivo situation and allow for a higher predictability of the situation in humans.
Here we demonstrate the suitability of primary cells for signaling assays in compound screenings using Clonetics™ and Poietics™ primary sensors, i.e. primary and stem cells expressing luminescent biosensor proteins specific for Ca^{2+} and cAMP. These assay formats are used on the FDSS®/µCELL plate reader to generate pharmacologically relevant data for GPCR ligands.

Materials and Methods

Detecting increases in intracellular concentrations of Ca^{2+} and cAMP

The primary sensors HUVEC calcium biosensor, HMVEC-L calcium biosensor used here transiently express the Ca^{2+} biosensor i-Photina® (Axxam) (see www.lonza.com/primarysensors). The GloSensor™ (Promega) was expressed alone in hMSC (hMSC cAMP biosensor) or co-expressed with i-Photina®. Transfection was carried out using the Amaxa™ Nucleofector™ technology.
The cells, provided frozen in vials, were thawed and seeded on 96-well or 384-well plates and allowed to recover over night in a humidified cell culture incubator. The i-Photina® expressing cells are ready-to-use preloaded with the substrate coelenterazine, GloSensor™ expressing cells need to be loaded with Promega's GloSensor™ cAMP reagent prior to the assay.

Agonists and antagonists were bought from Sigma-Aldrich or Calbiochem. Measurement of luminescence was carried out with the FDSS®/µCELL. Dose-dependent responses and EC/IC_{50} values were calculated using area under the curve (AUC) integration (GraphPad Prism®) for i-Photina® or using maximum peak values for GloSensor™.

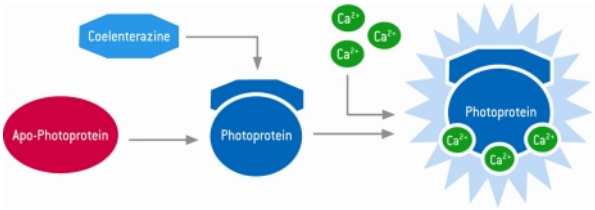


Fig. 1: Mechanism of the i-Photina reaction
Incubation of cells, expressing the i-Photina® apo-photoprotein, with coelenterazine in the presence of oxygen leads to formation of a stable complex, the active photoprotein. Calcium released from intracellular stores upon stimulation of the cells with agonists via G-protein coupled receptors binds to the photoprotein. The excited photoprotein converts coelenterazine to coelenteramide and emits a flash of blue luminescence.

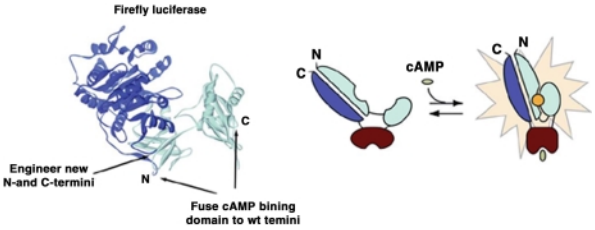


Fig. 2: GloSensor® Technology
Firefly luciferase has been fused to the cAMP-binding domain of human Protein kinase A (red). Upon binding of cAMP the whole protein molecule undergoes a conformational change. This activates the luciferase domain which converts luciferin to oxyluciferin and emits luminescence.
See www.promega.com/glosensor for more information on the live-cell blosensor.

Settings of the FDSS® /µCELL

Sensitivity was always set to 5, dispense height was 2 mm in all cases, dispense speed was 50 µL/sec for i-Photina® in 384-well and GloSensor™ (96-well only), 150 µL/sec for i-Photina® in 96-well. Detection interval was 2 sec for i-Photina®, 10 sec for GloSensor™.
Optimizing the settings included increasing the dispense speed from 50 to 150 µL/sec for i-Photina® in 96-well plate which led to increase in signal intensity. Altering dispense height in the range from 0.8 mm to 4 mm did not change luminescence intensity.
Slightly decreasing the detection area per well to 5×5 pixels for 384-well and 14×14 pixels for 96-well plates was necessary to reduce luminescence crossover to neighbouring wells on white plates.

Results

Specific stimulation and inhibition of GPCR-mediated signaling

The FDSS®/µCELL has been used to record flash luminescence as well as glow luminescence from different primary sensors in 96-well and 384-well assay formats. The FDSS®/µCELL offers the benefit of dispensing agonists into multiple samples in one step and recording the luminescence of all samples simultaneously.
The primary sensors assay system is well suited to generate pharmacologically relevant data for substances that trigger Ca^{2+}-dependent and cAMP-dependent signaling. Agonists for different classes of GPCRs (histamine receptors, purinergic receptors, β2-adrenergic receptors) clearly showed dose-dependent responses with the HUVEC and HMVEC-L calcium biosensor and the hMSC cAMP biosensor yielding EC_{50} values consistent with published data (Fig.3 & 4).
Z' factor was well above 0.5 for all three systems (data not shown).
The cAMP-mediated response to isoproterenol, an agonist of the β2-adrenergic receptor, could be specifically inhibited through propranolol, a known antagonist of this receptor, generating an IC_{50} (Fig.4) in line with published data.

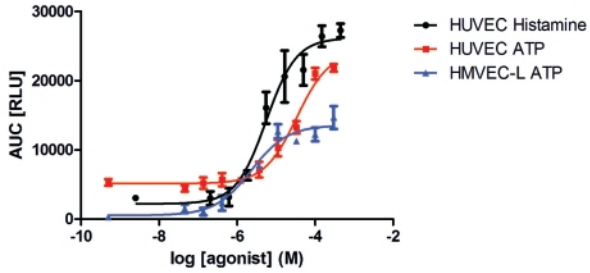


Fig. 3: HUVEC and HMVEC-L calcium biosensors respond to physiological ligands of cell surface GPCRs.

The assay was performed in a white 384-well plate as described in Materials and Methods. EC_{50} values (HUVEC: histamine 5.6 µM, ATP 34 µM; HMVEC-L ATP 2.3 µM) are consistent with published data.

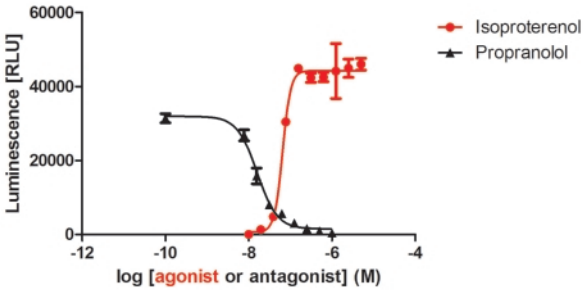


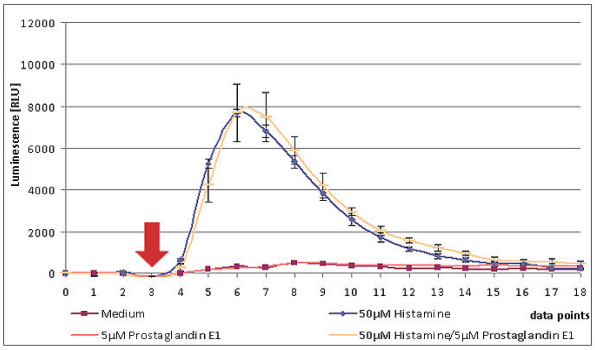
Fig. 4: Response of hMSC cAMP biosensor to GPCR ligand can be specifically inhibited by an antagonist.

The assay was carried out in white 96-well plate as described in Materials and Methods. Data calculated from maximum peak values. The IC_{50} value of 16 nM for propranolol and the EC_{50} of 65 nM for isoproterenol are in line with published data.

Consecutive detection of calcium and cAMP signaling in one sample

With hMSC co-expressing both biosensors it is possible to monitor Ca^{2+}-dependent and cAMP-dependent signaling in one sample. The cells were triggered simultaneously with two ligands specific for the histamine and for the prostaglandin E_1 receptor. The i-Photina® signal appears a few seconds after addition of the agonists and vanishes before the GloSensor™ luminescence rises which takes several minutes. As the two different luminescence signals appear consecutively they can easily be distinguished.

(A)



(B)

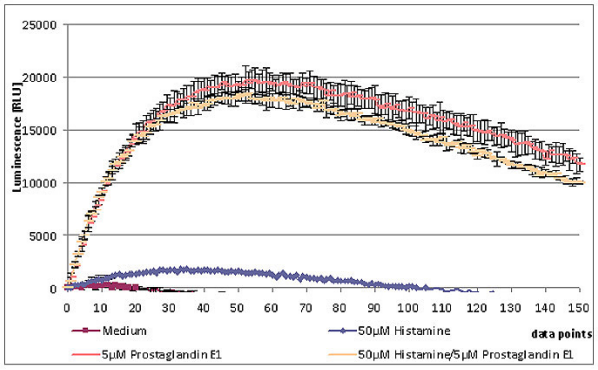


Fig. 5: Multiplexing detection of intracellular Ca^{2+} and cAMP levels in hMSC co-expressing both biosensors

The assay was carried out in a white 96-well plate as described in materials and methods. Two agonists were dispensed at the same time (indicated by red arrow) into each sample. i-Photina® flash luminescence was recorded immediately every 2 seconds for a total of 36 seconds (A), followed by measuring GloSensor™ glow luminescence every 10 seconds for 25 minutes (B).

Conclusion

Primary cells transiently transfected with either the calcium biosensor i-Photina®, the cAMP-sensitive GloSensor™ or co-transfected with both are available as ready-to-use tools for drug discovery research. They are a groundbreaking robust new assay system for detecting intracellular secondary messenger-dependent signaling upon stimulation with physiological and synthetic agonists. This was demonstrated for four different classes of G-protein coupled receptors in dose-dependent manners for two plate formats. Using the FDSS®/µCELL it has been possible to consecutively monitor on one instrument Ca^{2+}-dependent as well as cAMP-dependent signaling in one sample, simultaneously stimulated with the respective agonists. The FDSS®/µCELL reader is very well suited to reproducibly generate reliable pharmacologically relevant data with low variability from the Clonetics™ and Poietics™ primary sensors assay system.

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